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TITLE: PROTEINS OF HUMAN IMMUNODEFICIENCY VIRUS THAT CROSS-REACT WITH HUMAN "SELF" ANTIGENS

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13. ABSTRACT *(Maximum 200 words)*

AIDS is caused by the human immunodeficiency virus (HIV) and constitutes a major health hazard to military and civilian populations. Our goal with this contract is to understand pathogenesis and to obtain a rational strategy for developing a vaccine by dissecting the humoral and cellular immune responses to infection by HIV against both the virus and host (self) antigens. With our initial two year grant we defined the immunodominant domain of HIV-1 and HIV-2 for B cell (antibody) responses and fine mapped the minimal essential epitope to 7 amino acids segment: (aa603-609, HIV-1; aa597-603, HIV-2) both containing flanking cysteine residues. With the current (second) grant we have documented the essential contributions of the flanking cysteines and solved the two dimensional structure of this B cell epitope using NMR. Of murine monoclonal antibodies raised to HIV-1 Gp aa598-609, a few (7 of 51) also reacted with a novel astrocyte antigen. Conditions in which this novel antigen was expressed and the location in the brain where the greatest expression occurred were determined. Expression libraries were constructed and are under analysis for isolating the gene expressing this astrocyte antigen.

14. SUBJECT TERMS

RA 1; HIV; Immune responses; Immune cross-reactivity between viral and host proteins; CTL; Peptides; Novel astrocyte antigen; Expression library

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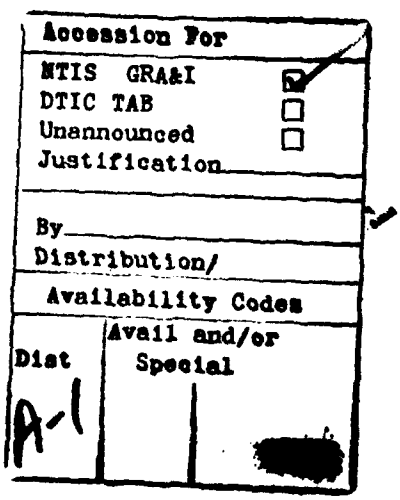
Cellular immune response to HIV was analyzed concurrently. Focusing on human cytotoxic T lymphocyte (CTL) responses to the P24 GAG protein of HIV, a number of novel CTL epitopes have been uncovered and mapped using synthesized peptides.



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_____In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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_____For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

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_____In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PI Signature Michael BA Oldstine Date 20 NOV 1991

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Mid-Term Scientific Report
Army Contract Number DAMD17-90-C-0070
Principal Investigator: Michael B. A. Oldstone, M.D.

Time Covered: 5/21/90 - 11/20/91

1. Introduction Overview

The acquired immunodeficiency syndrome (AIDS) is a global epidemic. Advances in diagnostic testing, understanding pathogenesis and vaccine development require a clear understanding of the humoral and cellular immune responses to infection by the human immunodeficiency virus (HIV), the etiologic agent of AIDS. Currently this infection constitutes a significant infectious disease health problem for both civilians and the military. Our research effort is on antigenic determinants on host (self antigens) cells and on HIV that are singularly or mutually recognized by antibodies and/or cytotoxic T lymphocytes (CTL) generated after infection or immunization with the virus. To that end we documented and fine mapped the immunodominant antibody domain of HIV-1 and of HIV-2 on GP41, aa603-609 and 597-603, respectively. Several monoclonal antibodies recognizing the 7-mer of HIV-1 were found to cross-react with the 7-mer of HIV-2 and vice versa. Interestingly, some of these monoclonal antibodies recognized a novel antigen on astrocytes of human, monkey, rat and mouse brains.

In this first half of support we used immunohistochemical staining and a number of experimental manipulations to show that the astrocyte antigen is not glial fibrillary acid protein (GFAP), is found on reactive astrocytes and although expressed widely throughout the central nervous system (CNS), can be manipulated to reach heightened intensity focally in the hippocampal region. This observation led us to construct a library from poly(A)$^+$ RNA isolated from hippocampi of mice expressing high concentrations of the novel astrocyte antigen as well as making a cDNA library from whole brain.

CTL from some HIV infected individuals react to a protein epitope on HIV-1 GP41 adjacent to and overlapping with the B cell (antibody) immunodominant domain we described. Utilizing proton nuclear magnetic resonance spectroscopy and several synthesized peptides from HIV-1 GP41 aa598-609, we fine mapped the peptide anatomy and conformation of this immunodominant domain. Owing to recent observations by B. Walker (unpublished data) that CTL are readily obtained from cerebral spinal fluid in HIV infected individuals and our observations that a vaccine containing only CTL but not B cell epitopes can protect mice from an acute and ordinarily lethal CNS infection (J. Virol. 63:4311, 1989) or prevent the establishment of a persistent viral infection (unpublished data), we began to look at the fine specificity of HIV-1 GAG CTL epitopes in collaboration with Dr. Frank Ennis. This allowed us to utilize GAG P24 overlapping peptides that we earlier synthesized for this Army contract as well as synthesizing new HIV-1 peptides for fine epitope mapping. With this collaboration and approach, two new HIV-1 P24 epitopes were identified and a number of others are currently under study.

2. Specific Accomplishments -- (Body of Scientific Report)

i. Anatomic map of the immunodominant domain (antibody) of HIV GP41 transmembrane protein: Peptide conformational analysis using synthesized peptides, monoclonal antibodies and proton nuclear magnetic resonance (NMR) spectroscopy

We have successfully completed the first of our three technical objectives proposed for this contract. Objective 1 (or A) was to determine the structure of the major immunodominant GP41 transmembrane protein (peptide epitope) of HIV-1. We proposed to determine whether or not recognition depended entirely on the flanking cysteines, the resultant disulfide bridge and lollipop configuration suspected to form this epitope.

As shown in Table 1, both the amino and carboxy termini cysteines of the HIV-1 and HIV-2 epitopes are essential for antibody binding. Both direct binding and cross-reactivity were clearly due to the conformation displayed by the 7-residue loop containing two cysteines, because disruption of the disulfide bond resulted in the loss of antibody recognition. This was observed with substitution of a serine (S) for a cysteine (C) at either the amino or carboxy end of the loop.

Table 1
Importance of the Cysteine (C)-Cysteine (C) Loop in the Immunodominant Epitope of HIV-1 and HIV-2 GP41 for Antibody Binding[1]

HIV-1 Peptides	End Point Dilution of MoAb to HIV-1:CSGKLIC[2]	HIV-2:CAFRQQVC[3]
CSGKLIC	126,000	1,400
SGKLIC	Nil	150
CSGKLIS	Nil	Nil
LGLWGSSGKLIC	1,000	140
HIV-2 Peptides		
CAFRQVC	680	34,000
SAFRQVC	Nil	Nil
CAFRQVS	Nil	Nil
NSWGSAFRQVC	Nil	400

[1]Normal mouse sera or ascites fluid were not positive against these peptides in a dilution >1/30. Values of 1/30 or less are recorded as Nil.

[2]Monoclonal antibody (MoAb) used was GP41-6.

[3]MoAb used was 115.8.

Further, the 7-mer peptide CSGKLIC, at a concentration of 20μg, blocked antibody binding to either the 12-mer of HIV-1 or the 11-mer of HIV-2 peptides. Immunochemical assays with dinitrophenol, a chemical commonly used to reduce disulfide bonds, were technically unsatisfactory due to solubility difficulties. The HIV GP41 transmembrane peptides have been used to generate monoclonal antibodies in mice and rats and antibodies in rabbits, to react with antibodies generated by almost all (>99%) humans during HIV infection and to react with CTL obtained from some HIV-1 infected persons indicating their immunogenicity as well as recognition by the immune system during natural infection.

Next, we collaborated with Dr. Peter Wright's group at Scripps to study the molecular conformation of the HIV-1 peptide in solution using NMR spectroscopy. The immunologically active form of CSGKLIC contained an intramolecular disulfide bond and maintained a preference for a conformation that included a type I reverse turn about residues SGKL. The preference was absent in the reduced form of the peptide that contained two thiol groups. Thus, the presence of the disulfide bond is integral to the formation of the structure of the loop in solution. These conclusions were reached by the experimentation displayed below (see Figures 1-4, Table 2) and supported by immunochemical data that elimination of loop formation by substitution of a serine (S) for a cysteine (C) at the amino or carboxy termini of the 7-mer is accompanied by the failure of antibody to bind to these peptides (see Table 1 above).

Conformation of reduced and oxidized CSGKLIC peptide were deduced from ^{1}H-NMR spectroscopy.

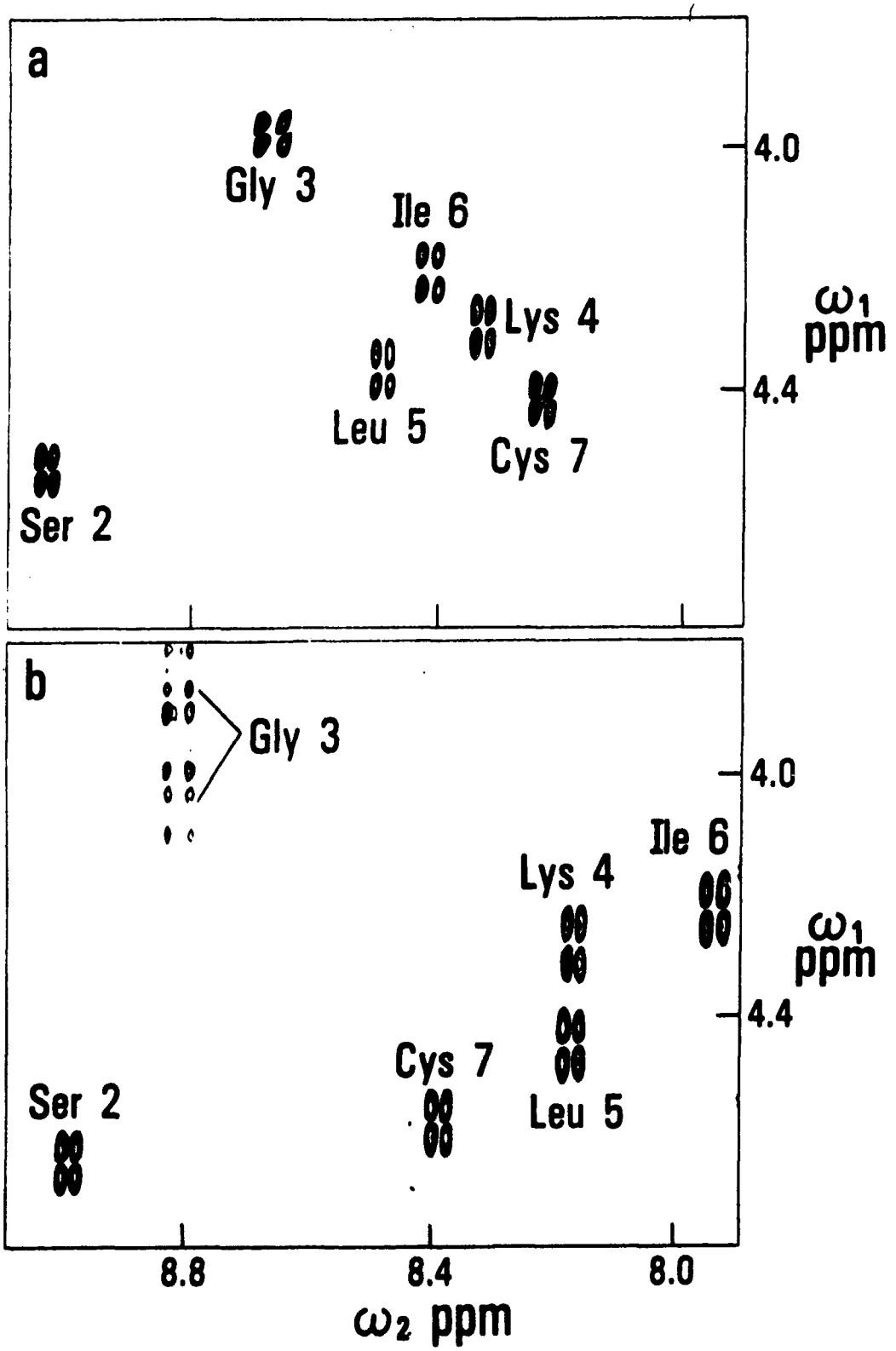

Figure 1: Fingerprint region of 2QF COSY spectra of reduced (a) and oxidized (b) heptamer. NH-C$^{\alpha}$H cross peaks are marked with the corresponding residue name. Numbers 2 through 7 correspond to residues 604 to 609 of the GP41 peptide representing the immunodominant epitope of HIV-1. The N-terminal residue of the peptide (Cys-1; residue 603) does not have an amide proton resonance.

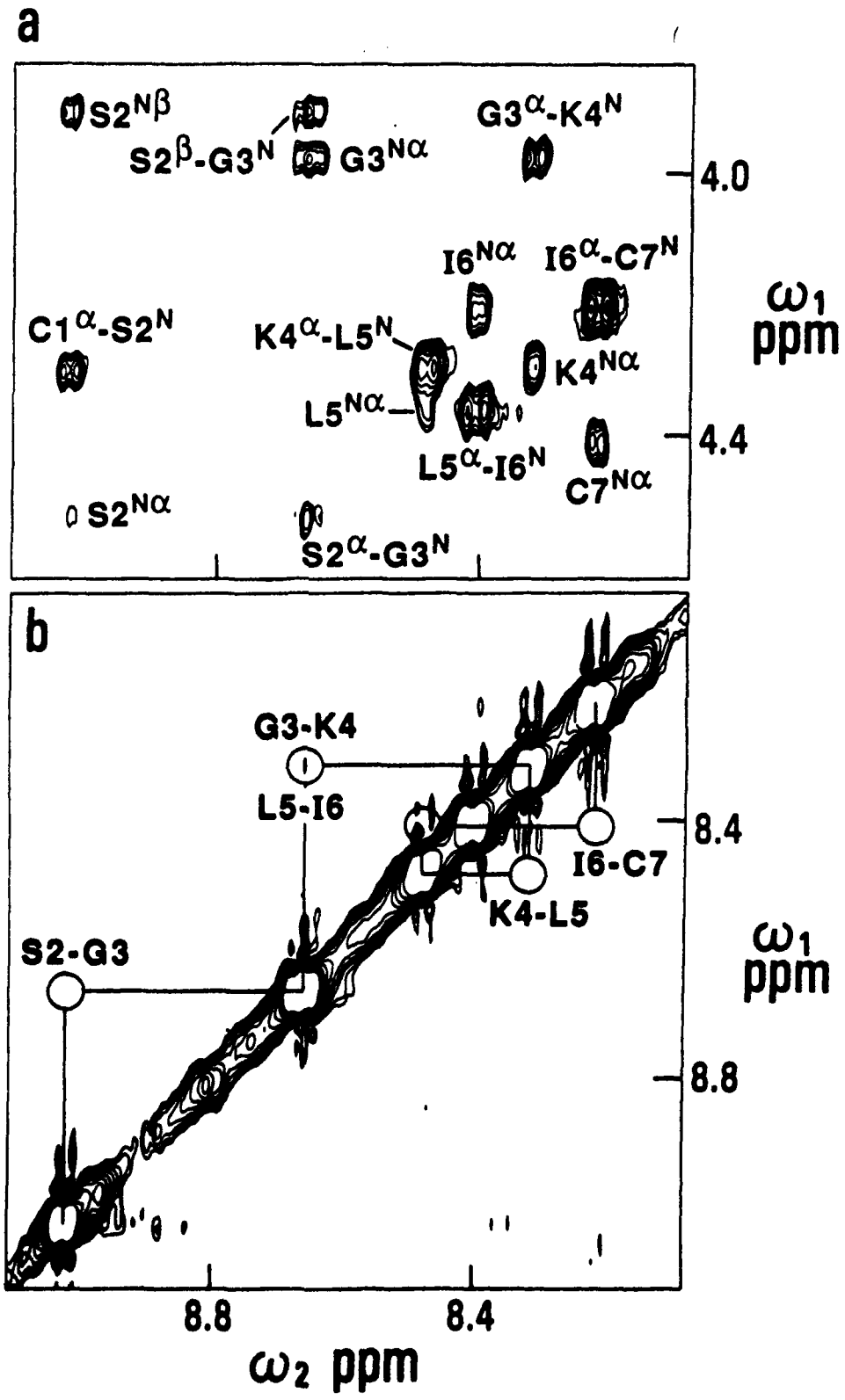

Figure 2: Portions of a 300-MHz ROESY spectrum (τ = 150 ms) of the reduced heptamer at 7278 K, pH 3.80. (a) NH-α region. Sequential NOE cross peaks between the C$^{\alpha}$H of each residue and the NH of the subsequent residue are marked, as well as the corresponding peaks for the intraresidue interaction (Figure 1A). (b) NH-NH region. Diagonal corresponds to a one-dimensional NMR spectrum. Positions of sequential $d_{NN}(i,i+1)$ NOE cross peaks (not observed in this spectrum) are indicated.

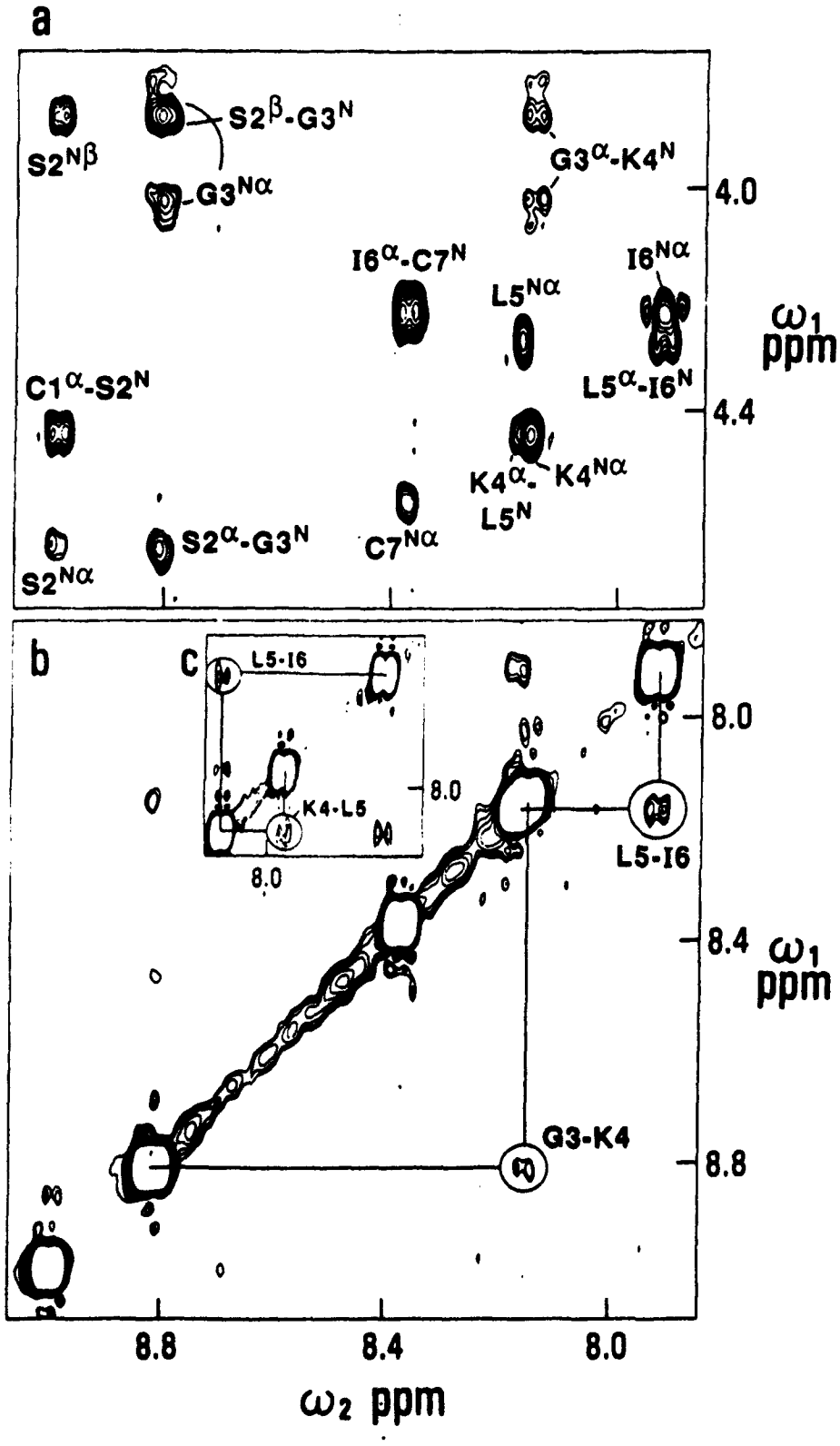

Figure 3: Portions of 300-MHz ROESY spectra (τ =100 ms of oxidized heptamer at 278 K. (a) NH-α region. (b) NH-NH diagonal region. (Inset, c) Region of a 300-MHz ROESY spectrum at 298 K (τ - 150 ms).

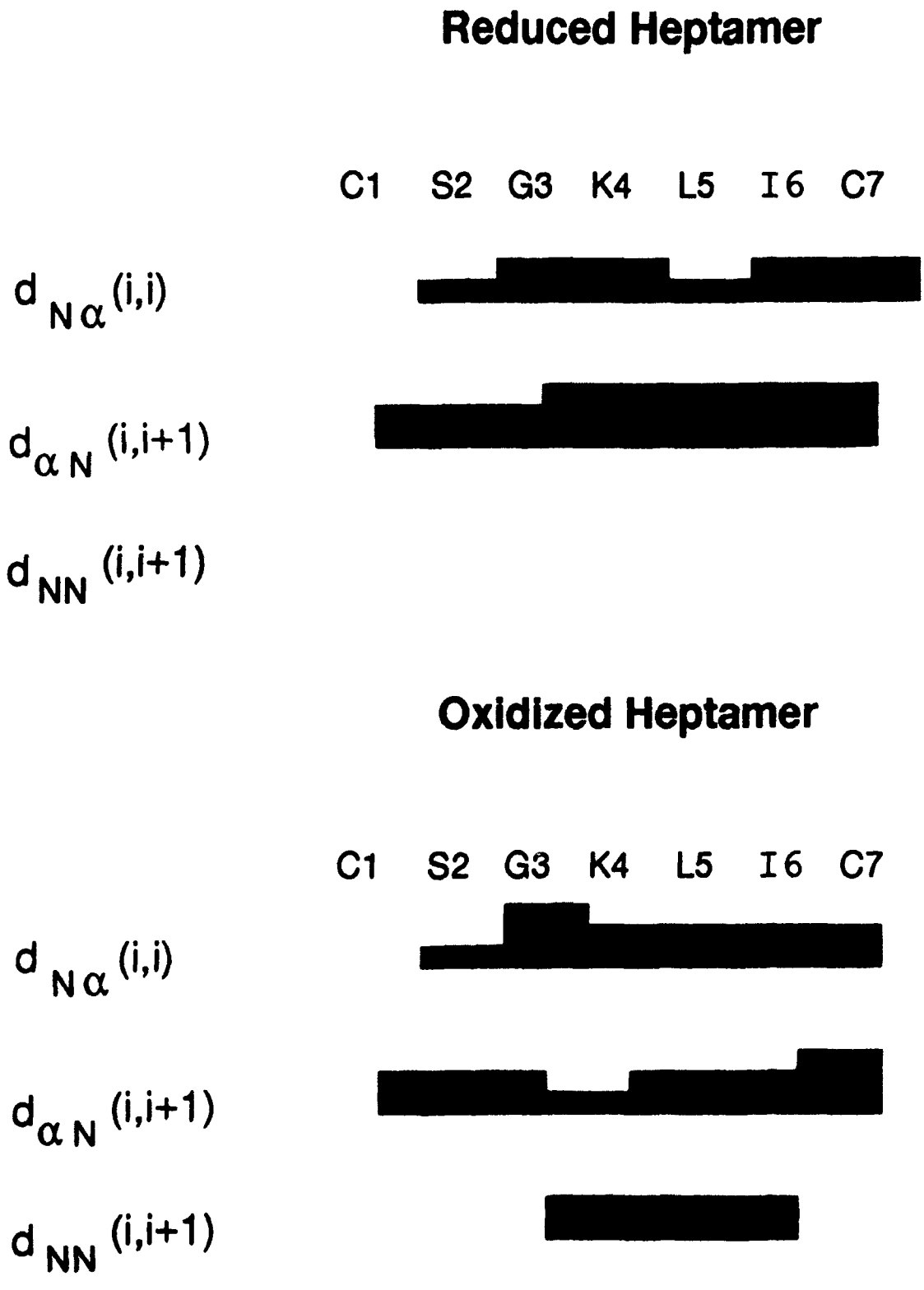

Figure 4: Schematic diagram summarizing the NOE connectivities observed in the NMR spectra of the reduced and oxidized heptamer peptides.

Table 2
Resonance Assignments, Temperature Coefficients, and $^3J_{HN\alpha}$ Coupling Constants for Oxidized and Reduced Heptamer

Residue no.	Chemical shift (ppm) (278 K, pH 3.8)						$-\Delta\delta/\Delta T$, 10^3 (ppm/K)[a]	$^3J_{HN\alpha}$ (Hz)
	NH	C$^\alpha$H	C$^\beta$H	C$^\gamma$H	C$^\delta$H	C$^\epsilon$H		
Reduced heptamer (dithiol)								
Cys-1		4.30	3.15					
			3.08					
Ser-2	9.03	4.53	3.92				6.7	6.30
Gly-3	8.67	3.98					8.3	NM[b]
Lys-4	8.33	4.30	1.71	1.4	1.6	3.0	7.5	6.69
			1.78		1.7			
Leu-5	8.49	4.37	1.62	1.67	0.94		8.3	6.63
					0.88			
Ile-6	8.41	4.21	1.91	1.50	0.86	0.93 ($C^\gamma H_3$)	10.1	8.04
				1.22				
Cys-7	8.23	4.41	2.92				8.0	7.59
Oxidized heptamer (disulfide)								
Cys-1		4.47	3.18					
			3.38					
Ser-2	9.01	4.67	3.86				7.4	6.75
			3.88					
Gly-3	8.83	3.86					7.6	NM
		4.08						
Lys-4	8.19	4.48	1.79	1.42	1.73	3.04	8.8	7.80
			1.83	1.22				
Leu-5	8.19	4.31	1.72	1.66	0.96		3.4	6.60
			1.76		0.92			
Ile-6	7.96	4.26	1.88	1.22	0.89	0.94 ($C^\gamma H_3$)	9.5	8.61
				1.51				
Cys-7	8.42	4.61	3.28				9.1	7.47
			3.09					

[a] Temperature coefficient of amide proton chemical shift = gradient of linear plot of chemical shift (δ) versus temperature (T). Since δ decreases with increasing T, the gradient has a negative sign.
[b] NM, Not measured.

ii. Analysis of the novel astrocyte ("self") antigen (M22) recognized by monoclonal antibody generated against the HIV-1 GP41 immunodominant domain and the construction of cDNA libraries that contain the gene expressing this astrocyte epitope.

This comprised technical objective 2 (or B) on the contract proposal and we have made considerable inroads in this area. The unique astrocytic antigen was initially noted to possess a putative molecular weight of 43kd (J. Virol. 65:1370, 1991) and to be recognized by several monoclonal antibodies made against HIV-1 GP41 peptide LGLWGCSGKLIC (J. Virol. 65:1727, 1991). Because the monoclonal antibody with the highest titer and lowest background that recognized these reactive astrocytes was MAb 22 (Figure 5), we have tentatively termed the astrocyte antigen M22.

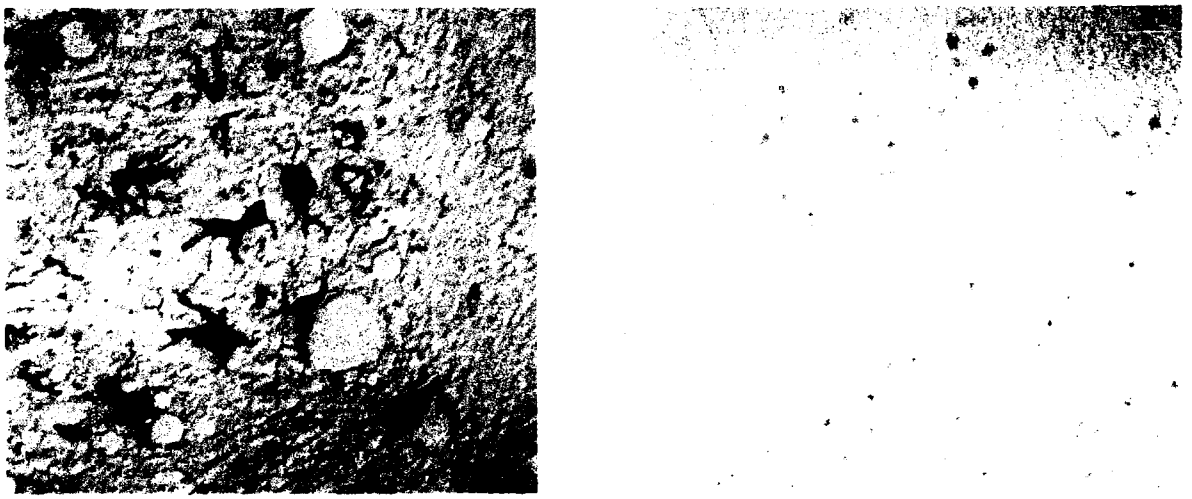

Figure 5

Figure 5: Immunohistochemistry of the M22 antigen in the hippocampus in reactive astrocytes. Double-labelling with an anti-GFAP polyclonal sera indicated that $M22^+$ cells were astrocytes (data not shown). M22 antigen is rarely expressed in astrocytes from normal brains as displayed in the negative panel also obtained from a corresponding hippocampal area.

Our initial observations (J. Virol. 65:1370, 1991) documented the M22 antigen in reactive astrocytes surrounding a cerebral vascular lesion in human tissue and in SJL/J mice infected with Theiler's virus or developing allergic encephalomyelitis. Despite extensive study of astrocyte lines with a wide battery of manipulations we were unable to detect the M22 antigen in cultured cells. Hence, the first cDNA expression library was made from CNS of Theiler's virus infected SJL/J mice. While a few positive plaques were noted with M22 monoclonal antibody, these were in limited abundance and could not be enriched on subsequent serial passages. For that reason we abandoned the first cDNA expression library and undertook experiments to enrich for the M22 antigen. First, we evaluated a number of mouse strains and noted the greatest M22 antigen expression occurred with SWR/J and C57Bl/6 mice followed by BALB > SJL/J. We then evaluated a number of experimental conditions in order to have high M22 antigen expression. We found that scrapie agent caused, by several fold, the highest expression of M22 antigen (scrapie > Theiler's = EAE). Lastly, we evaluated different regions in the CNS of SWR/J and C57Bl/6 mice for M22 expression and reactive astrocytosis. As seen in Figure 6, the greatest expression of RNA (for GFAP) or M22 antigen (by immunohistochemistry, data not shown) occurred in the hippocampal region.

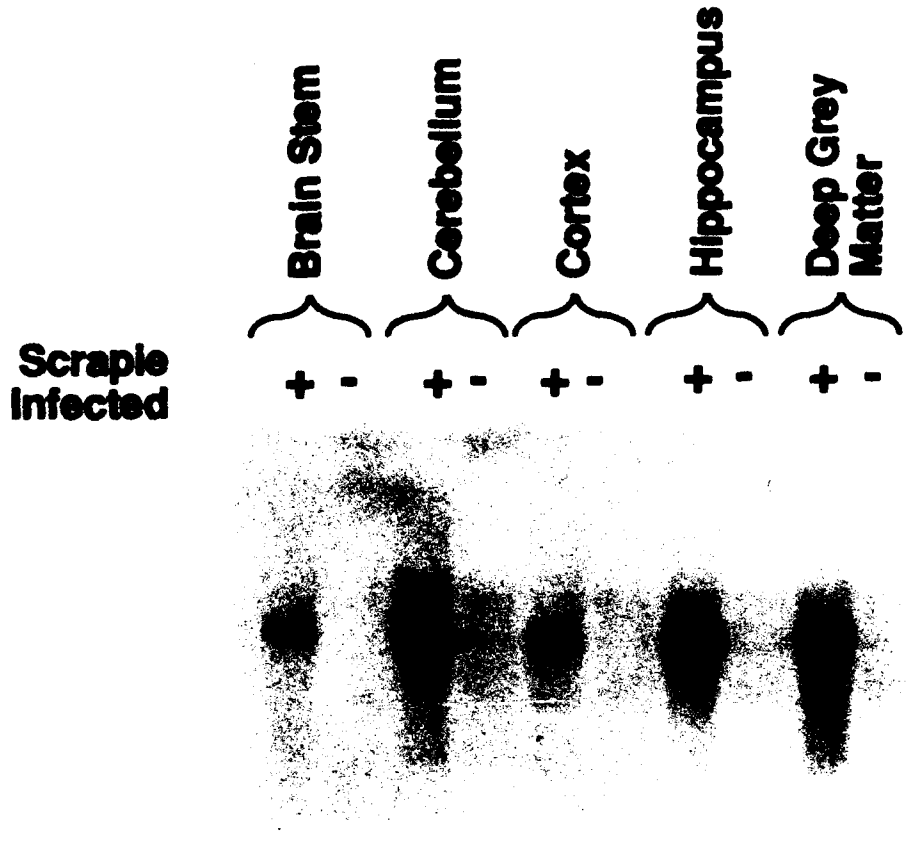

Figure 6: Northern blot analysis of the quality and GFAP expression in total RNA isolated from control (-) and scrapie-infected (+) brain regions in C57Bl/6 mice. A large increase in the level of GFAP RNA can be seen in all the infected brain regions; the band is discrete indicating the high quality of the RNA. The RNA transfer blot contains 10μg of total RNA isolated from the respective brain regions in each lane and was hybridized with the GFAP cDNA labelled with ^{32}P by the random primer method. Similar findings occurred with SWR/J mice inoculated with scrapie agents.

Armed with the above data we have just successfully constructed two new cDNA libraries. One is from the total CNS of Swiss NIH (SWR/J like) mice infected with scrapie agent while the second is from the hippocampal region of C57Bl/6 mice infected with scrapie agent. In the first instance, three male Rocky Mountain Laboratory Swiss mice (RML Swiss, Rocky Mountain Laboratories, Hamilton, MN) inoculated with scrapie agent were killed by cervical dislocation, their brains removed rapidly, snap frozen in liquid nitrogen and stored at 70°C. The brain tissues were pooled for isolation of the RNA. Poly(A)$^+$ RNA was isolated using the Bradley procedure (Biotechniques 6:114, 1988). First strand cDNA was synthesized from poly(A)$^+$ RNA by reverse transcription with oligo (dT) primers. RNase H, E.Coli DNA ligase and DNA polymerase I were used for synthesis of the second strand, and T4 DNA polymerase was used to blunt the ends of the double-stranded cDNA. BstXI non-palindromic linkers were ligated onto the cDNA and the product ligated into pcDNA II. The library contains $5x10^5$ individual recombinants.

To make a cDNA library from the hippocampus, eight male C57Bl/6J mice inoculated with scrapie agent were killed, brains removed and dissected into 5 regions: the cerebral neocortex, cerebellum, hippocampal formation, brain stem and deep grey matter (made up predominantly of the thalamus and hypothalamus). These regions were pooled and snap frozen in liquid nitrogen and stored at 70°C. Total RNA was isolated using the acid guanidinium thiocyanate-phenol chloroform method (Chomczynski and Sacchi, Analytical Biochem 162:156, 1987). The quality of the RNA was checked at this stage by northern blot hybridization. 10μg of total RNA from each pooled region and from control, uninfected regions were resolved on a formaldehyde denaturing agarose gel and blotted onto a nylon membrane. A fragment of mouse GFAP cDNA was labelled to 10^9cpm/μg of DNA using random primers and hybridized to the blot. The RNA was found to be of good quality. Poly(A)$^+$ RNA was then selected with one cycle of oligo (dT)-cellulose chromatography. 4μ of poly(A)$^+$ RNA from each infected and uninfected region was again run on a Northern Blot, blotted and probed with GFAP. As above, this verified that the RNA quality was good. First and second strand cDNA was synthesized as above and the cDNA ligated into pcDNA II. This library is currently being titered.

To insure sufficient supply of monoclonal antibody to M22 for screening, we obtained over 600cc of ascites fluid. The antibody was titered using the procedure documented in Figure 5 and shown to have a negligible tissue background but a specific M22 antigen titer of greater than 1:10,000 dilution with detection observed at 1:20,000 dilution. We are now positioned to use this antibody to screen our libraries. In addition we will use the new antibody to try to purify sufficient M22 antigen to attempt N-Terminal sequencing in order to make an oligonucleotide probe. If successful this probe will also be of assistance in screening our libraries. Objective 3 (or C) is to use the gene to express sufficient amounts of the M22 antigen to allow its biochemical and structural analysis as well as evaluating its potential role in HIV-1 pathogenesis. This component will begin on the successful conclusion of Objective 2 (or B).

iii. Mapping peptide epitopes on HIV-1 GAG that are recognized by CTL generated by HIV infected individuals

On agreement with Colonel Burke we began a collaboration with Dr. Frank Ennis (Professor of Medicine, Division of Infectious Diseases, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655) to use GAG overlapping peptides that we synthesized with funds from the first 2 year contract to map HIV CTL. The HIV CTL was first identified using a vaccinia-GAG recombinant, the CTL then cloned and expanded in culture for study. Peptides that overlap HIV-1 GAG P24 were sent to Ennis to test against the CTL found to react against vaccinia virus/GAG recombinant. The initial results using our 20 amino acid peptides that overlapped the P24 region of HIV-1 GAG identified a CD8$^+$ CTL clone that recognized aa140-152.

Table 3

Peptide Synthesized	CTL Lysis (CD8$^+$, pt 1)	CTL Lysis (CD4$^+$, pt 2)
GAG HIV aa 133-152	68%	15%
146-152	<1%	<1%
138-152	65%	17%
140-152	62%	20%
133-148	9%	14%
138-148	10%	15%
140-148	3%	16%

Interestingly, this GAG region of aa140-152 is conserved by 17/18 (94%) of sequenced HIV-1 isolates. The one divergent strain shows a single aa change of valine to isoleucine at residue 143. The data also

documented overlap in CTL epitope recognition by the $CD8^+$ clone and a $CD4^+$ clone (Table 3). However, aa149-142 was not required for the $CD4^+$ CTL clone's recognition while in contrast aa149-152 was required for $CD8^+$ CTL recognition (Table 3).

To precisely define the CTL epitope, numerous peptides were made with single amino dilutions at the amino or at the carboxy termini of HIV GAG P24 aa140-152. Further, peptide aa140-152 with either an isoleucine or a valine at residue 143 was synthesized. By this methodology we mapped a unique epitope on the GAG protein to aa145-150. The CTL clone was $CD8^+$ and the restricting MHC glycoprotein was HLA-Cw3. This was the first instance of HLA-C restriction for a human CTL response and interestingly like the GP41 immunodominant domain initially studied by our group, the CTL epitope for GAG 145-150 overlaps with a major B cell epitope of GAG (J. Virol. 65:4051, 1991). The data for peptide epitope mapping of this HLA-Cw3 restricted $CD8^+$ CTL clones is given below in Table 4

Table 4

GAG Peptide Residue	Amino Acid Sequence[1]	Percent Specific Lysis[2]
aa 133-152	PIVQNIQGQMVHQAISPRTL	68%
146-152	AISPRTL	<1%
138-152	IQGQMVHQAISPRTL	65%
140-152	GQMVHQAISPRTL	62%
133-148	PIVQNIQGQMVHQAIS	10%
138-148	IQGQMVHQAIS	11%
140-148	GQMVHQAIS	3%
140-152	GQMVHQAISPRTL	37%
145-151	QAISPRT	13%
145-150	QAISPR	25%
146-152	AISPRTL	<1%

[1]P24 GAG sequence based on HIV-1 (IIIB) strain sequence.
[2]Autologous target-CTL assay. Effector to target cell ration was 4:1.

We then turned our attention to the $CD4^+$ CTL clone. The clone was restricted by HLA DQ and fine mapped to GAG aa140-148 (Table 5). Thus, the peptides of HIV GAG P24 recognized by this $CD4^+$ CTL clone overlaps with the $CD8^+$ CTL described above. This epitope is also highly conserved among all recorded HIV-1 isolates (J. Virol., in press 1992). The data for the peptide mapping is given below in Table 5.

Table 5

GAG Peptide Residue	Amino Acid Sequence	Percent Specific Lysis
aa 133-152	PIVQNIQGQMCHQAISPRTL	15%
146-152	AISPRTL	<1%
138-152	IQGQMVHQAISPRTL	17%
140-152	GQMVHQAISPRTL	20%
133-148	PIVQNIQGQMVHQAIS	14%
138-148	IQGQMVHQAIS	15%
140-148	GQMVHQAIS	17%

Additionally, when this $CD4^+$ CTL clone was incubated with either purified P24 protein, or peptides aa140-152, 141-152 or 140-148, cytokines IL-2 and IL-6 were synthesized while in contrast neither IL-4, γinterferon nor TNFα were made.

3. Summary of Mid-Term Report

The structure of the HIV-1 GP41 immunodominant domain was analyzed using synthesized peptides, monoclonal antibodies to the domain, and proton nuclear magnetic resonance spectroscopy. The immunologically active epitope is mapped to aa603-609 = CSGKLIC. This peptide contains an intramolecular disulfide bond and maintains a preference for a folded conformation including a type I reverse turn around residues SGKL. No such preference is observed for the reduced form of the peptide, which contains two thiol groups. The presence of the disulfide bond is integral to the formation of the structure of the loop. Elimination of loop formation by substitution of S for C at the amino or carboxy termini of the 7-mer is accompanied by the failure of antibody binding to this peptide.

Antibody (monoclonals) and antibody from HIV-1 infected individuals that bind to this 7-mer peptide also bind to a novel epitope on reactive astrocytes termed M22 antigen. The M22 antigen has been found on reactive astrocytes in human, monkey, rat and mouse brains. Heightened concentrations of M22 antigen was found in the hippocampal region of brains from Swiss (SWR/J like) or C57Bl/6 mice inoculated with scrapie agent. This observation provided a substrate in which M22 antigen and presumably mRNA is enriched. Expression cDNA libraries were constructed from Poly(A^+) RNA obtained from the hippocampal area of C57Bl/6 mice inoculated with scrapie and from whole brains of SWR/J mice also inoculated with scrapie. These libraries are being processed and screened to obtain the gene expressing the M22 antigen.

GAG P24 epitopes have been mapped for a $CD8^+$ and a $CD4^+$ CTL clone. The $CD8^+$ CTL clone is restricted by HLA-Cw3 and recognizes residues 145 to 150 QAISPR. The $CD4^+$ CTL clone is restricted by HLA-DQ and binds to residues 140-148 GQMVHQAIS. Hence, the $CD8^+$ and $CD4^+$ CTL recognize four common amino acids. Both these CTL epitopes' amino acid sequences are highly conserved among HIV-1 strains. The $CD4^+$ CTL clone release IL-2 and IL-6 but not γinterferon or TNFα upon stimulation with peptide aa140-148 or purified P24 protein. CTL have now been shown to commonly be in the cerebral spinal fluid of HIV-1 infected individuals. The roles of CTL and cytokines as well as antibodies and CTL that recognize both HIV-1 determinants and host "self" antigen determinants (molecular mimicry) in the pathogenesis of AIDS is a matter of current and future investigations.

PUBLICATIONS

A. Listed in press previously, now published

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C. Abstracts

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